

Adaptor-related proteins

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Two new adaptor-related protein complexes, AP-3 and AP-4, have recently been identified, and both have been implicated in protein sorting at the *trans*-Golgi network (TGN) and/or endosomes. In addition, two families of monomeric proteins with adaptor-related domains, the GGAs and the stoned B family, have also been identified and shown to act at the TGN and plasma membrane, respectively. Together with the two conventional adaptors, AP-1 and AP-2, these proteins may act to direct different types of cargo proteins to different post-Golgi membrane compartments.

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Current Opinion in Cell Biology 2001, 13:444–453

0955-0674/01/\$ — see front matter

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Abbreviations

AP adaptor protein

ARF ADP-ribosylation factor

GGA Golgi-localized, γ ear-containing, ARF-binding protein

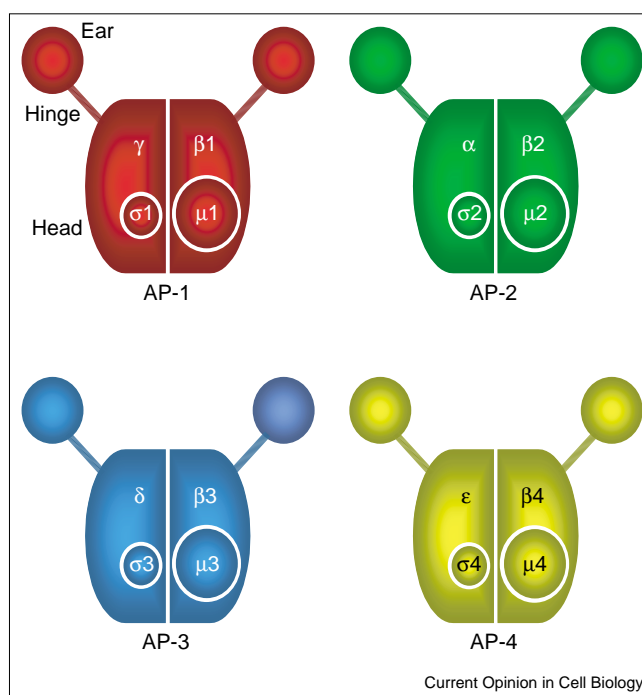
TGN *trans*-Golgi network

Introduction

The AP-1 and AP-2 adaptor protein (AP) complexes are components of clathrin-coated vesicles associated with the *trans*-Golgi network (TGN) and plasma membrane, respectively. They attach the clathrin to the membrane, select the vesicle cargo and recruit accessory proteins that regulate vesicle formation. Both adaptor complexes are heterotetramers, with two large subunits (γ and $\beta 1$ in the AP-1 complex, α and $\beta 2$ in the AP-2 complex), a medium-sized or μ subunit and a small or σ subunit. The two complexes are believed to have similar structures, resembling Mickey Mouse, with a core or 'head' consisting of the medium and small subunits and the amino-terminal domains of the two large subunits, flanked by two 'ears' consisting of the carboxy-terminal domains of the two large subunits, connected by flexible hinges (Figure 1).

The different subunits of the AP complexes perform different functions. The β subunits are particularly important for clathrin binding [1,2], and clathrin-binding consensus sequences (L[L,I][D,E,N][L,F][D,E]) have been identified in the hinge domains of both $\beta 1$ and $\beta 2$ [3]. In addition, there is some evidence that the β ears [4] and the hinge domains of the γ and α subunits [5,6] also contribute to clathrin binding. The μ and β subunits have been implicated in cargo selection. Three distinct sorting signals for selection into clathrin-coated vesicles have been identified in the

Figure 1



Schematic diagrams of the four AP complexes. All four complexes consist of two large subunits: a β subunit and a more divergent subunit, either γ , α , δ or ϵ ; a medium (μ) subunit; and a small (σ) subunit. The carboxy-terminal domains of the two large subunits project as 'ears', connected to the 'head' of the complex by flexible hinges. Yeast two-hybrid experiments have shown that the $\gamma/\alpha/\delta/\epsilon$ subunits interact with the σ subunits, that the β subunits interact with the μ subunits and that the two large subunits interact with each other.

cytoplasmic tails of certain transmembrane proteins: NPXY, YXX \emptyset (where \emptyset is a bulky hydrophobic residue) and dileucine [7]. Of these, the best characterized is the YXX \emptyset signal, and this sequence binds to the μ subunits of both AP-1 and AP-2 [8,9]. Crosslinking studies suggest that dileucine signals bind to the β subunits [10]. The α and β ears [4,11], and presumably the γ ear as well [12], recruit accessory proteins onto the membrane, where they participate in events such as vesicle scission and vesicle uncoating.

The search for novel adaptors

Proteins containing NPXY, YXX \emptyset and dileucine sorting signals can have very different steady state distributions in the cell and can be localized at the plasma membrane, recycling endosomes, late endosomes, lysosomes and the TGN, as well as at more specialized organelles such as melanosomes and synaptic vesicles. Studies from a number of laboratories have demonstrated that these sorting signals not only act as internalization signals when the proteins are at the plasma membrane, they also help to

determine the proteins' trafficking itineraries and steady state distributions inside the cell [7]. Thus, it became apparent several years ago that only two adaptors, one for TGN to endosome traffic and one for plasma membrane to endosome traffic, are not sufficient to explain all the other post-Golgi sorting events that use NPXY, YXXØ or dileucine signals.

The hypothesis that there must be additional adaptors or adaptor-related proteins prompted the search for homologues of known AP subunits. Although early strategies involving PCR or low stringency library screening proved to be unsuccessful, over the past five years the explosion of new DNA sequence data, particularly from the human EST (expressed sequence tag) database, has made it possible to search by computer for novel members of each of the four adaptor subunit families. With the complete sequencing of the *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster* and human genomes, we now know of the existence of three types of adaptor-related proteins. First, some of the AP-1 and AP-2 subunits exist as two or more distinct isoforms, encoded by different genes. It has long been known that in mammals there are two isoforms of the AP-2 α subunit, although no clear-cut functional differences between them have been reported [13]. More recently it has been found that in mammals there are also two isoforms of the AP-1 γ , $\mu 1$ and $\sigma 1$ subunits. At present the functional significance of the two γ and $\sigma 1$ subunits is unclear [14,15]; however, the second $\mu 1$ subunit, $\mu 1B$, has been well characterized. Unlike the other AP-1 and AP-2 subunit genes, which are all expressed ubiquitously, $\mu 1B$ is specifically expressed in epithelial cells [16], and it plays an important role in trafficking to the basolateral plasma membrane [17,18].

In addition to the novel isoforms of known subunits, which (as far as we know) can assemble into variants of the AP-1 and AP-2 complexes, there are also two new complete complexes, AP-3 and AP-4. These complexes are also heterotetramers consisting of two large subunits, a medium subunit and a small subunit (Figure 1). Finally, two families of monomeric adaptor-related proteins with homology to AP subunit domains have been identified: the GGA family and the stoned B family. This review will focus on AP-3, AP-4, the GGAs and stoned B.

AP-3

The first AP-3 component to be identified was its μ subunit, which was found somewhat serendipitously in an expression library screen. Two isoforms of this protein, p47A and p47B, were identified in rat that had distinct expression profiles, with p47A (later renamed $\mu 3A$) expressed ubiquitously and p47B ($\mu 3B$) expressed only in neurons and neuroendocrine cells [19]. Later, an isoform of the AP-3 β subunit was reported, β -NAP (for neuronal adaptin-like protein) [20], and a subsequent study revealed that p47 and β -NAP could be coimmunoprecipitated, indicating that they are part of the same complex [21]. The

remaining subunits of the complex, δ (homologous to γ and α) and $\sigma 3$ (two isoforms, both expressed ubiquitously), as well as a ubiquitously expressed β subunit ($\beta 3A$, with β -NAP renamed $\beta 3B$), were all found in the EST database [22–25]. Antibodies against the subunits showed that they all associate with each other and that they localize to the TGN and to a more peripheral compartment that partly colocalizes with endosomal markers [21–23].

AP-3 mutants

Studies on AP-3 function were greatly facilitated by the discovery of naturally occurring AP-3 mutants in *Drosophila*, mouse and humans, and by the creation of AP-3 mutants in yeast. Four of the classical *Drosophila* eye colour mutants, *garnet*, *ruby*, *carmine* and *orange*, are in genes encoding the AP-3 δ , $\beta 3$, $\mu 3$ and $\sigma 3$, subunits respectively [23,25–29]. These belong to the granule group of genes, which also includes *Drosophila* homologues of the yeast vacuolar protein sorting (*VPS*) genes *VPS18* (deep orange), *VPS33* (carnation) and *VPS41* (light). The phenotype of the granule group of eye colour mutants, together with the discovery that some of the granule group genes have yeast *VPS* gene homologues, suggested that all of these genes, including those encoding AP-3 subunits, might be involved in trafficking to lysosomes and related organelles [30].

This hypothesis was confirmed when mammalian AP-3 mutants were identified. Two mouse models for the human genetic disorder Hermansky-Pudlak syndrome (HPS), *mocha* and *pearl*, have mutations in the AP-3 δ and $\beta 3A$ subunits, respectively [31,32*]. Subsequently, two human HPS patients with mutations in the $\beta 3A$ gene were identified [33*]. Both the mice and the humans have defects in lysosomes and lysosome-related organelles, in particular in their melanosomes and platelet dense granules, leading to a number of problems including hypopigmentation, prolonged bleeding and pulmonary fibrosis. Remarkably, however, especially in the case of the *mocha* mouse, which has a null mutation in the δ subunit and no functional AP-3 whatsoever [31], both the mice and the humans survive to adulthood. This is in marked contrast to mice with AP-1 subunit knockouts, which are embryonic lethal [34,35].

AP-3-mediated trafficking

Although AP-3 is clearly involved in trafficking to lysosomes, the precise pathway that it mediates is still not clear. The major missorting defect that has been observed in AP-3 deficient mammalian tissue culture cells is that certain lysosomal membrane proteins, including lamp-1, lamp-2, limp-2 and CD63, show increased trafficking via the plasma membrane, although their steady state distribution is still mainly lysosomal [33*,36,37]. When AP-3 subunits are deleted in yeast, two vacuolar membrane proteins, alkaline phosphatase and Vam3p, are missorted, although the vacuolar hydrolase carboxypeptidase Y (CPY) is sorted correctly [38,39]. In contrast, most of the classical yeast *vps* mutants, which were originally identified as

defective in CPY sorting, sort alkaline phosphatase correctly, indicating that different molecules use different pathways to get to the vacuole. The observation that *vps* mutants that specifically affect trafficking through a pre-vacuolar compartment are still able to sort alkaline phosphatase has led to the proposal that the AP-3 pathway takes proteins directly from the late Golgi to the vacuole or lysosome, bypassing any sort of endosomal intermediate [40]. One problem with this hypothesis, when applied to mammalian cells, is that much of the AP-3 is not localized to the TGN but to a more peripheral endosomal compartment [3,21–23]. However, it is possible that the AP-3 pathway uses a different endosomal intermediate from the ‘conventional’ pathway.

It is not clear why certain organelles, such as melanosomes and platelet dense granules, are more strongly affected than lysosomes in AP-3 deficient mammals. This may be because of more extreme missorting of some of the proteins that normally reside in these organelles or alternatively because organelles may be more sensitive to subtle differences in sorting efficiency. Other mouse models for HPS that have a similar overall phenotype, including pale ear (the mouse orthologue of the human *HPS1* gene), do not show the characteristic rerouting of lysosomal membrane proteins to the plasma membrane [37], indicating that the proteins encoded by these genes are not components of the AP-3 vesicle budding machinery, but probably act at some other trafficking step. The genes mutated in the pallid and gunmetal mouse strains have recently been cloned and encode a syntaxin-13-interacting protein and the rab geranylgeranyltransferase α subunit respectively [41,42], indicating that they facilitate vesicle docking and fusion rather than vesicle budding.

Another question is whether the AP-3 vesicles are clathrin-coated. The AP-3 β 3A and β 3B subunits both contain clathrin-binding consensus sequences in their hinge/ear domains, and both bind to clathrin *in vitro* [3]. In addition, the clathrin amino-terminal domain has been cocrystallized with a β 3A subunit derived clathrin-binding peptide [43], and AP-3 recruits clathrin assembly onto synthetic liposomes [44]. However, AP-3 is not enriched in purified clathrin-coated vesicles [20,21], and deleting AP-3 subunit genes in yeast has a completely different phenotype from deleting clathrin heavy or light chain genes [38]. Furthermore, an *in vitro* system that reconstitutes the budding of synaptic-like microvesicles from PC12 cell endosomes is dependent on AP-3 but apparently does not require clathrin [45]. Double labelling immunolocalization studies give variable results depending on the antibody [3,21,23]. Methods that do not rely on the use of antibodies, such as live cell imaging of cells coexpressing AP-3 and clathrin subunits coupled to different fluorescent proteins, should help to resolve this dispute.

It seems clear that in yeast, at least, the AP-3 pathway does not require clathrin, and consistent with this finding, yeast

β 3 does not contain clathrin-binding consensus sequences. The best candidate for a protein that might be taking the place of clathrin in yeast is Vps41p, one of the few *VPS* gene products involved in the trafficking of alkaline phosphatase as well as CPY. Vps41p resembles the clathrin heavy chain in that it contains potential WD40 domains and a clathrin heavy chain repeat, and it interacts with the carboxy-terminal hinge/ear domain of the yeast AP-3 δ subunit [46•]. However, Vps41p is also implicated in the docking and fusion of vesicles at the vacuole, suggesting that it may act at both the budding and the fusion step [47•]. A Vps41p homologue has been identified in mammals [48], so it will be important to characterize this protein and determine whether or not it colocalizes with AP-3.

AP-3 in neurons

Another unresolved issue concerns the function of AP-3 in neurons. It is striking that two of the AP-3 subunits have neuronal-specific isoforms and that the mocha mouse, which lacks functional AP-3 complexes in all of its tissues, has severe behavioural abnormalities, whereas the pearl mouse, which can assemble AP-3 complexes in its neurons because of the presence of β 3B, does not [31]. The requirement for AP-3 in synaptic-like microvesicle budding from endosomes also suggests that AP-3 has a specific role to play in neurons, especially since only the neuronal-specific form of AP-3 is active in the *in vitro* budding assay [49]. However, ultrastructural studies of the mocha mouse [31] and of AP-3 deficient flies [29], do not show any obvious abnormalities in the nerve terminals, suggesting that the absence of AP-3 only affects the biogenesis of a specialized population of synaptic vesicles. It is clear that there is much that we do not understand about AP-3 function; however, the availability of AP-3 mutants in flies and mammals gives us the unique opportunity of being able to study the consequences of AP-3 deficiency in living multicellular organisms.

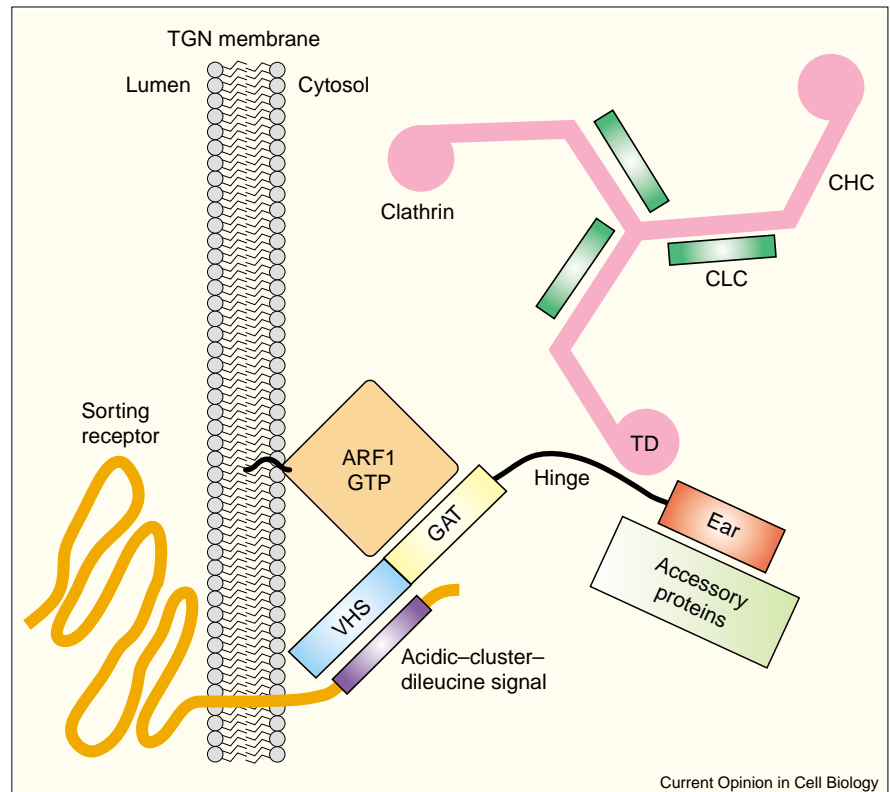
AP-4

The first hint to the existence of a fourth adaptor protein complex came from the identification of a novel human protein termed μ -ARP2 that was related to the μ subunits of AP complexes [50]. Later studies identified three additional human proteins named ϵ , β 4 and σ 4 that were homologous to the $\alpha/\gamma/\delta$, β and σ subunits, respectively, of AP complexes [51•,52•]. Biochemical analyses showed that ϵ , β 4, σ 4 and μ -ARP2 (renamed μ 4) were components of a novel heterotetrameric complex designated AP-4 ([51•,52•]; Figure 1).

The phylogeny of the AP-4 complex differs from that of the other three AP complexes. Genes encoding AP-4 subunits or closely related homologues have been identified in organisms belonging to the animal (humans, mice and chickens), plant (*Arabidopsis thaliana*) and protist (*Dictyostelium discoideum*) kingdoms. Intriguingly, some animals (*C. elegans* and *Drosophila*) and protists (*S. cerevisiae*) lack orthologues of AP-4 subunits altogether. The

Figure 2

Schematic representation of the GGAs and their interaction partners. The GGAs comprise four domains designated VHS, GAT, hinge and ear. The VHS domain (blue) of the mammalian GGAs binds acidic-cluster-dileucine signals present in the cytoplasmic domains of sorting receptors such as the cation-dependent mannose 6-phosphate receptors and sortilin. The GAT domain (yellow) is responsible for the recruitment of the GGAs to the TGN, which is at least in part due to interaction with the GTP-bound form of ARF. The hinge domain (black) mediates interactions with the terminal domain (TD) of the clathrin heavy chain (CHC), whereas the ear domain (red) binds accessory proteins thought to regulate coat formation or vesicle budding. CLC, clathrin light chain. Adapted from [71].



evolutionary and physiological significance of this unusual pattern of expression remains to be elucidated.

In mammals, the four subunits of AP-4 are expressed in all tissues and cells examined [51•,52•]. Immunofluorescence microscopy analyses revealed that the AP-4 complex is largely associated with the TGN, as demonstrated by its colocalization with the TGN markers, TGN38 and furin [51•,52•]. Treatment of cells with brefeldin A caused dissociation of AP-4 from the TGN into the cytosol [51•,52•], suggesting that recruitment of AP-4 to membranes, like recruitment of AP-1, AP-3 and coatomer, is regulated by a member of the ADP-ribosylation factor (ARF) family of GTP-binding proteins. Immunoelectron microscopy further refined the localization of AP-4 to non-clathrin-coated vesicles in the area of the TGN [52•]. Consistent with the latter finding, AP-4 could not be detected in preparations of clathrin-coated vesicles [52•]. Moreover, the hinge domains of the ϵ and $\beta 4$ subunits lack consensus motifs for clathrin binding present in other clathrin-associated proteins. These observations suggest that AP-4 is most probably part of a non-clathrin coat.

The low abundance of the AP-4 complex in most cells has so far hampered further characterization of the structures or molecules with which AP-4 associates *in situ*. Yeast two-hybrid and *in vitro* binding assays have demonstrated that $\mu 4$ binds YXX ϕ -type tyrosine-based sorting signals from

TGN38 [53] and the lysosomal membrane proteins lamp-1 [53], lamp-2 [54] and CD63 [52•], albeit with low affinity. Screening of a combinatorial peptide library using the yeast two-hybrid system showed that $\mu 4$ has characteristic preferences for residues surrounding the critical tyrosine, the most notable being phenylalanine at positions -1 and $+3$ relative to the tyrosine [54]. The physiological meaning of these preferences is still unclear. Placement of a $\mu 4$ -specific tyrosine-based sorting signal onto the cytoplasmic tail of a plasma membrane reporter protein resulted in targeting of the chimera to the endosomal-lysosomal system [54], suggesting a role for AP-4 in this process. A more definitive assessment of AP-4 function, however, will probably require ablation of genes encoding AP-4 subunits in genetically tractable organisms such as mice or *D. discoideum*.

GGAs

The GGAs (Golgi-localized, γ ear-containing, ARF-binding proteins) were identified independently by a number of groups using different approaches, including, searching through DNA databases for novel proteins with homology to adaptor subunits [55•–57•] or containing a VHS domain (see below) [58•], screening a yeast two-hybrid library for proteins that bind to activated ARF [59•] or looking for genes in yeast that accentuate the growth defects caused by temperature-sensitive clathrin heavy chain [60•]. Although the GGAs have a carboxy-terminal domain homologous to the γ -adaptein ear, they are otherwise different from γ -adaptein and other

adaptor subunits. They appear by both gel filtration and ultracentrifugation to be monomeric [55•,56•], and they have a unique four-domain structure consisting of a VHS (Vps27p, Hrs, STAM) domain, a GAT (GGA and TOM1) domain, a hinge-like domain and an ear domain (Figure 2).

GGA mutants

Like the heterotetrameric adaptor complexes, the GGAs are highly conserved, and their genes have been identified in mammals, *S. cerevisiae*, *Drosophila* and *C. elegans*. Three GGA genes are ubiquitously expressed in mammals, and their three protein products colocalize at the TGN [55•–57•]. Insights into GGA function came initially from studies using yeast. *S. cerevisiae* expresses two GGA genes, *GGA1* and *GGA2*, both of which need to be deleted to get a clear-cut phenotype (deleting *GGA2* alone gives a weak phenotype, but this is thought to be because it is expressed at 5–10 fold higher levels than *GGA1* [60•]). Vacuolar protein sorting is moderately affected in GGA-deficient yeast: more than 50% of the CPY is secreted instead of retained intracellularly, and the electrophoretic mobility of the secreted CPY indicates that it has been incompletely processed [55•,56•,61•]. The GGAs are also required for normal sorting of the prevacuolar syntaxin Pep12p [62•] and the α -factor processing enzyme Kex2p, which normally cycles back and forth between the late Golgi and an endosomal compartment [63•]. Together, these observations point to a role for the GGAs in TGN to endosome trafficking.

GGA domains

Further insights into GGA function have come from studies of their four individual domains and their binding partners. At the amino terminus of all of the GGAs is a VHS domain. VHS domain-containing proteins have been extensively studied and the crystal structures of two VHS domains have been solved [64,65], but until recently nothing was known about VHS domain function. However, two groups have now independently shown that the VHS domains of mammalian GGAs bind to the cytoplasmic domains of three proteins that traffic from the TGN to endosomes: sortilin, the cation-independent mannose 6-phosphate receptor and the cation-dependent mannose 6-phosphate receptor [66•,67•,68]. The TGN to endosome sorting signal for all three proteins is a cluster of negatively charged amino acids followed by a dileucine [66•,69•], and this motif is also responsible for binding to GGAs [66•,67•,68]. Overexpressing truncated GGA constructs consisting of just the VHS and GAT domains causes mannose 6-phosphate receptors to become trapped in the TGN [67•,70,71]. Other dileucine signals implicated in trafficking are not preceded by acidic clusters and presumably are sorted differently (e.g., by binding to the β subunit of the AP-1 complex [10]). In addition, different GGA VHS domains have different binding preferences [67•], and VHS domains from other proteins, and even from yeast GGAs, are likely to participate in other types of protein–protein interactions, as other mammalian VHS domains are unable to bind the acidic-cluster-dileucine

motif [67•] and none of the yeast proteins that are sorted by GGAs appears to have this type of signal. The sequence FSDSPEF facilitates GGA-mediated sorting of Pep12p in yeast and thus is a good candidate for another type of VHS-domain-binding partner [62•].

Downstream from the VHS domain is the GAT domain. The GAT domain is responsible for ARF binding in both mammalian and yeast GGAs [56•,59•,61•], and in mammalian cells it is both necessary and sufficient for localising the GGAs to Golgi membranes [56•]. Mutations in the GAT domain that abolish ARF binding also prevent such constructs from being recruited onto the membrane [71•]. Once the GGA has bound to the ARF, ARF-GAP activity is inhibited, stabilizing the association of the ARF–GGA complex with the membrane [71•]. When GAT domains are expressed at extremely high levels, other coats whose membrane association is ARF-dependent become cytosolic, presumably because all the available ARF is occupied by the GAT constructs [56•,71•]. All of these findings indicate that ARF binding and membrane recruitment are intimately coupled. However, there must be additional factors involved in the recruitment of GGAs onto membranes, because the distribution of GGAs is normally restricted to the TGN, whereas other ARF-dependent coats can have quite different distributions including the *cis*-Golgi/intermediate compartment and endosomes.

The hinge domain is the least well conserved among the GGAs in both length and amino acid sequence. However, its amino acid content is similar to that of the adaptin hinge domains, indicating that it too may be a flexible linker connecting the amino- and carboxy-terminal domains. In addition, all of the GGA hinge domains contain potential clathrin-binding sequences. Initial reports suggested that the GGAs might not be clathrin-associated, primarily because they were not detectable in purified clathrin-coated vesicles [55•]. However, it was subsequently shown that when cells are broken open the GGAs detach from the membrane, which could account for their absence from such preparations [63•]. More recent studies have shown that GGAs and clathrin interact *in vitro* [71•], that they colocalize at both the light and the electron microscope level in mammalian cells [63•,71•], that GGAs can recruit clathrin onto membranes [71•], that GGAs and clathrin act cooperatively in yeast [60•] and that GGAs and clathrin can be coimmunoprecipitated from yeast cell extracts [60•]. The identification of the GGAs as potential clathrin adaptors helps to explain the apparent paradox that in yeast it is possible to delete genes encoding subunits from all three AP complexes without affecting clathrin-coated vesicle formation or clathrin-mediated protein sorting [70,72,73,74].

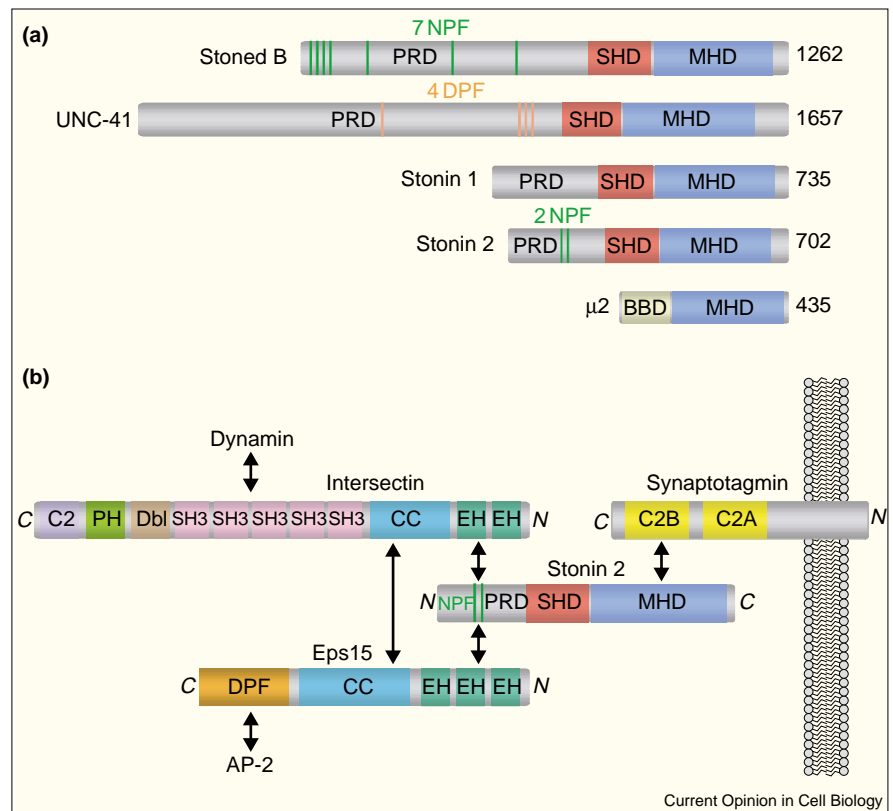
At the carboxy-terminal end is the γ -adaptin ear homology domain. By analogy with the α -adaptin ear, it is likely that both the GGA ear and the γ ear recruit accessory proteins onto the membrane. So far, only a single binding partner has been definitively identified for the γ ear, the EH

Figure 3

Schematic representation of members of the stoned B family and their interactions with components of the endocytic machinery.

(a) The scheme depicts the domain organization of *Drosophila* stoned B, *C. elegans* UNC-41, human stonins 1 and 2, and the mammalian μ 2 subunit of the AP-2 complex. The different domains are designated proline-rich domain (PRD), stonin-homology domain (SHD), μ -homology domain (MHD) and β -binding domain (BBD). The approximate positions of NPF motifs and the number of amino acids in each protein are indicated.

(b) Human stonin 2 is shown interacting with a member of the synaptotagmin family of protein via MHD–C2B domain interactions and with intersectin and Eps15 via NPF–EH domain interactions. The SH3 domains of intersectin in turn mediate an interaction with dynamin, whereas a domain containing multiple DPF motifs in Eps15 mediates interactions with the α -adaptin subunit of AP-2. CC, coiled-coil domain; Dbl, disabled; PH, pleckstrin homology domain.



domain-containing protein γ -synergins [12]. The function of γ -synergins is still unknown, although it can bind to the cytoplasmic domain of SCAMP1, an integral membrane that shuttles between the plasma membrane, endosomes and the TGN [75]. γ -synergins also shows limited binding to the GGA ears *in vitro* [55[•],57[•]], and colocalization of γ -synergins and GGAs has been reported in cells expressing tagged versions of both proteins [57[•]]. However, colocalization between endogenous GGAs and γ -synergins does not appear to be significant [57[•]], and, in addition, γ -synergins coimmunoprecipitates with the AP-1 complex but not with GGAs [12,55[•]]. GST pulldowns indicate that the GGA ears bind to at least three other proteins that also bind to the γ ear, and identification of these proteins is currently under way [55[•]]. Somewhat surprisingly, however, deletion studies in yeast indicate that although the VHS and GAT domains are absolutely required for GGA function, GGAs lacking their ear domains are at least partially functional [63[•]]. Replacing the GGA ear with the γ -adaptin ear fully restores function [63[•]], suggesting that in yeast as well as in mammals, the GGA and γ ears share some of the same binding partners.

Together, these studies indicate that the GGAs are functionally very similar to conventional clathrin adaptors, but they are monomeric instead of heterotetrameric. In the heterotetrameric adaptor complexes, cargo selection,

membrane localization, clathrin binding and accessory protein recruitment have been attributed to distinct subunits, whereas in the GGAs these same four functions appear to be performed by the VHS, GAT, hinge and ear domains respectively. In yeast, deleting AP-1 subunit genes together with GGA genes exacerbates the missorting phenotype, even though deleting AP-1 on its own has no effect, indicating that the two adaptors can to some extent substitute for each other [60[•],63[•]].

The importance of the GGAs in TGN to endosome trafficking has now been established beyond any doubt, and the ability of the GGAs to interact with cargo that had previously been assumed to use the AP-1 pathway raises the question of why the cell needs both types of adaptors. Possible explanations include different cargo preferences, different acceptor compartments (e.g., the AP-1 pathway may be from the TGN to early endosomes and the GGA pathway from TGN to late endosomes) [62[•]], different donor compartments (e.g., different subdomains of the TGN) or even different directions (e.g., the GGAs may facilitate traffic out of the TGN whereas AP-1 may be more important for retrograde traffic back to the TGN) [35].

Stoned B and related proteins

The founding member of another family of adaptor-related proteins is a product of the *Drosophila* stoned gene termed

stoned B ([76]; Figure 3a). Stoned mutants were originally isolated in a screen for temperature-sensitive paralytic flies, which exhibited uncoordinated wing and leg movements indicating neurological dysfunction [77]. The *stoned* gene displays synthetic lethal interactions with *shibire*, another paralytic gene that encodes a dynamin homologue involved in endocytosis at the synapse [77,78*]. In addition, presynaptic terminals of stoned mutants display impaired uptake of the dye FM1-43 [78*,79*] and depletion of synaptic vesicles [80]. All of these observations indicate that the neurological deficiencies of *stoned* mutants are probably due to defects in endocytic recycling of synaptic vesicle membranes.

The molecular characterization of *stoned* gene products provides additional support for their possible involvement in endocytosis. Strikingly, the *stoned* gene produces a dicistronic message encoding two proteins termed stoned A and stoned B [76]. Stoned A has no homology to any other known protein, although it contains five DPF (single amino acid code) motifs [76,81] that could potentially interact with the ear domain of α -adaptin [82]. Stoned B, on the other hand, exhibits limited homology to the signal-binding domain of AP μ subunits ([76]; Figure 3a). A homologue of stoned B is encoded by the *C. elegans unc-41* gene, mutations of which also result in an uncoordinated phenotype ([80]; Figure 3a). In addition, two homologues of stoned B, termed stonin 1 and stonin 2, have been recently described in humans and mice ([83,84*]; Figure 3a). No homologues exist in *S. cerevisiae*. The dicistronic *Drosophila* stoned mRNA is most abundant in the head, indicative of preferential expression in the central nervous system [76]. The human stonin 1 and stonin 2 mRNAs, on the other hand, seem to be uniformly expressed in most tissues and cells [84*].

All members of the stoned B family share a similar modular structure, consisting of a variable amino-terminal domain enriched in proline and serine residues (i.e., proline-rich domain or PRD), a conserved domain specific to this family (i.e., stonin-homology domain or SHD) and a carboxy-terminal domain homologous to the signal-binding domain of the μ subunits of AP complexes (i.e., μ -homology domain or MHD) (Figure 3a). Although not conserved at the amino acid sequence level, the PRDs of some of these proteins contain potential binding sites for components of the clathrin endocytic machinery. For example, the proline-rich domains of *Drosophila* stoned B [80] and human stonin 2 (but not stonin 1) [84*] contain seven and two NPF motifs, respectively. Thus, of the two human stonins, stonin 2 is the one that most resembles *Drosophila* stoned B. NPF (single amino acid code) motifs interact with EH domains of proteins such as eps15, γ -synergism and intersectins [75,85]. In fact, human stonin 2 interacts directly with eps15 and intersectin 1 via NPF-EH domain interactions [84*]. These interactions allow indirect linkage of stonin 2 to AP-2 [84*]. The PRD of the *C. elegans* homologue lacks NPF motifs but contains four DPF

motifs that could potentially mediate direct binding to the ear domain of the α subunit of AP-2 [82].

Despite the presence of a μ -homology domain in stoned B and related proteins, stonin 1 and stonin 2 are not part of AP complexes, nor do they appear to bind canonical tyrosine-based or dileucine-based sorting signals [84*]. Instead, the μ -homology domains of both stoned B [86*] and stonin 2 [84*] bind members of the synaptotagmin family of proteins, more specifically to their C2B domains. Synaptotagmins are membrane-anchored proteins proposed to function as docking sites for AP-2 via interactions with the AP-2 α and μ 2 subunits [87,88]. By analogy, synaptotagmins could recruit stonin 2 to the plasma membrane (Figure 3b). In turn, stonin 2 could serve as a linker for recruitment of eps15, intersectins and their respective binding partners (e.g., AP-2, dynamin and clathrin) (Figure 3b). Overexpression of stonin 2 in non-neuronal cells impairs recruitment of AP-2 to the plasma membrane and receptor-mediated endocytosis [84], providing additional evidence for the involvement of stonin 2 with the endocytic machinery. Thus, stonin 2 and AP-2 appear to participate in similar (and perhaps common) networks of protein interactions contributing to the assembly of the endocytic machinery at the plasma membrane.

Recent studies of *Drosophila* stoned mutants have suggested an alternative explanation for the physiological role of synaptotagmin-stoned B-interactions. These studies have shown that synaptotagmin I is mislocalized and destabilized in *stoned* mutants [80]. In addition, transgenic overexpression of synaptotagmin I in *stoned* flies restores viability and endocytic trafficking to normal levels [79*]. These observations suggest that a major role of stoned B may be to recruit or maintain synaptotagmin I at sites of endocytosis so that it can carry out its roles in synaptic vesicle biogenesis.

Conclusions

Over the past few years, some of our previous ideas about clathrin-coated vesicles have had to be modified. Originally it was assumed that the only components of the coats were clathrin and adaptor complexes, either AP-1 or AP-2. Now we know that there are a number of adaptor-associated proteins that regulate clathrin-coated vesicle formation. We also know that there are two additional types of adaptor complexes, AP-3 and AP-4, which are closely related to AP-1 and AP-2, even though they may be able to function independently of clathrin. Two novel monomeric proteins with adaptor-like domains have also been identified: GGAs, which may function as clathrin adaptors in their own right, and stoned B and its homologues, which probably participate in the AP-2 pathway. These new adaptor-related proteins and complexes all have distinct cargo preferences, and they also for the most part have distinct distributions within the cell; thus, they could potentially be used to sort different types of cargo on different post-Golgi trafficking pathways. Further studies will be needed to identify the full set of cargo molecules sorted by each adaptor-related protein, as well as to establish the exact subcellular

localization of these proteins and the molecular mechanisms in which they are involved. Eventually, this will lead to an integrated view of how adaptors and adaptor-related proteins control protein sorting in the late secretory and endocytic pathways.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Ahle S, Ungewickell E: **Identification of a clathrin-binding subunit in the HA2 adaptor protein complex.** *J Biol Chem* 1989, **264**:20889-20093.
2. Shih W, Gallusser A, Kirchhausen T: **A clathrin binding site in the hinge of the β 2 chain of mammalian AP-2 complexes.** *J Biol Chem* 1995, **270**:31083-31090.
3. Dell'Angelica EC, Klumperman J, Stoorvogel W, Bonifacino JS: **Association of the AP-3 complex with clathrin.** *Science* 1998, **280**:431-434.
4. Owen DJ, Vallis Y, Pearse BM, McMahon HT, Evans PR: **The structure and function of the β 2-adaptin appendage domain.** *EMBO J* 2000, **19**:4216-4227.
5. Goodman OB, Keen JH: **The α chain of the AP-2 adaptor is a clathrin binding subunit.** *J Biol Chem* 1995, **270**:23768-23773.
6. Morgan JR, Prasad K, Hao W, Augustine GJ, Lafer EM: **A conserved clathrin assembly motif essential for synaptic vesicle endocytosis.** *J Neurosci* 2000, **20**:8667-8676.
7. Bonifacino JS, Dell'Angelica EC: **Molecular bases for the recognition of tyrosine-based sorting signals.** *J Cell Biol* 1999, **145**:923-926.
8. Ohno H, Stewart J, Fournier MC, Bosshart H, Rhee I, Miyatake S, Saito T, Gallusser A, Kirchhausen T, Bonifacino JS: **Interaction of tyrosine-based sorting signals with clathrin-associated proteins.** *Science* 1995, **269**:1872-1875.
9. Owen DJ, Evans PR: **A structural explanation for the recognition of tyrosine-based endocytic signals.** *Science* 1998, **282**:1327-1332.
10. Rapoport I, Chen YC, Cupers P, Shoelson SE, Kirchhausen T: **Dileucine-based sorting signals bind to the beta chain of AP-1 at a site distinct and regulated differently from the tyrosine-based motif-binding site.** *EMBO J* 1998, **17**:2148-2155.
11. Slepnev VI, De Camilli P: **Accessory factors in clathrin-dependent synaptic vesicle endocytosis.** *Nat Rev Neurosci* 2000, **1**:161-172.
12. Page LJ, Sowerby PJ, Lui WWY, Robinson MS: **γ -Synergisin: an EH domain-containing protein that interacts with γ -adaptin.** *J Cell Biol* 1999, **146**:993-1004.
13. Ball CL, Hunt SP, Robinson MS: **Expression and localisation of α -adaptin isoforms.** *J Cell Sci* 1995, **108**:2865-2875.
14. Takatsu H, Sakurai M, Shin F, Murakami K, Nakayama K: **Identification and characterization of novel clathrin adaptor-related proteins.** *J Biol Chem* 1998, **273**:24693-24700.
15. Lewin DA, Sheff D, Ooi CE, Whitney JA, Yamamoto E, Chicone LM, Webster P, Bonifacino JS, Mellman I: **Cloning, expression, and localization of a novel γ -adaptin-like molecule.** *FEBS Lett* 1998, **435**:263-268.
16. Ohno H, Tomemori T, Nakatsu F, Okazaki Y, Aguilar RC, Foelsch H, Mellman I, Saito T, Shirasawa T, Bonifacino JS: **μ 1B, a novel adaptor medium chain expressed in polarized epithelial cells.** *FEBS Lett* 1999, **449**:215-220.
17. Fölsch H, Ohno H, Bonifacino JS, Mellman I: **A novel clathrin adaptor complex mediates basolateral targeting in polarized epithelial cells.** *Cell* 1999, **99**:189-198.
18. Fölsch H, Pypaert M, Schu P, Mellman I: **Distribution and function of AP-1 clathrin adaptor complexes in polarized epithelial cells.** *J Cell Biol* 2001, **152**:595-606.
19. Pevsner J, Volkhardt W, Wong BR, Scheller RH: **Two rat homologs of clathrin-associated adaptor proteins.** *Gene* 1994, **146**:279-283.
20. Newman LS, McKeever MO, Hirota OJ, Darnell RB: **β -NAP, a cerebellar degeneration antigen, is a neuron-specific vesicle coat protein.** *Cell* 1995, **82**:773-783.
21. Simpson F, Bright NA, West MA, Newman LS, Darnell RB, Robinson MS: **A novel adaptor-related protein complex.** *J Cell Biol* 1996, **133**:749-760.
22. Dell'Angelica EC, Ohno H, Ooi CE, Rabinovich E, Roche KW, Bonifacino JS: **AP-3: an adaptor-like protein complex with ubiquitous expression.** *EMBO J* 1997, **16**:917-928.
23. Simpson F, Peden AA, Christopoulou L, Robinson MS: **Characterization of the adaptor-related protein complex, AP-3.** *J Cell Biol* 1997, **137**:835-845.
24. Dell'Angelica EC, Ooi CE, Bonifacino JS: **β 3A-adaptin, a subunit of the adaptor-like complex AP-3.** *J Biol Chem* 1997, **272**:15078-15084.
25. Ooi CE, Moreira JE, Dell'Angelica EC, Poy G, Wassarman DA, Bonifacino JS: **Altered expression of a novel adaptin leads to defective pigment granule biogenesis in the *Drosophila* eye color mutant garnet.** *EMBO J* 1997, **16**:4508-4518.
26. Lloyd VK, Sinclair DA, Wennberg R, Warner TS, Honda BM, Grigliatti TA: **A genetic and molecular characterization of the garnet gene of *Drosophila melanogaster*.** *Genome* 1999, **42**:1183-1193.
27. Mullins C, Hartnell LM, Wassarman DA, Bonifacino JS: **Defective expression of the μ 3 subunit of the AP-3 adaptor complex in the *Drosophila* pigmentation mutant carmine.** *Mol Gen Genet* 1999, **262**:401-412.
28. Kretzschmar D, Poock B, Roth H, Ernst R, Keller A, Porsch M, Strauss R, Pflugfelder GO: **Defective pigment granule biogenesis and aberrant behavior caused by mutations in the *Drosophila* AP-3 β -adaptin gene ruby.** *Genetics* 2000, **155**:213-223.
29. Mullins C, Hartnell LM, Bonifacino JS: **Distinct requirements for the AP-3 adaptor complex in pigment granule and synaptic vesicle biogenesis in *Drosophila melanogaster*.** *Mol Gen Genet* 2000, **263**:1003-1014.
30. Lloyd V, Ramaswami M, Kramer H: **Not just pretty eyes: *Drosophila* eye-colour mutations and lysosomal delivery.** *Trends Cell Biol* 1998, **8**:257-259.
31. Kantheti P, Qiao X, Diaz ME, Peden AA, Meyer GE, Carskadon SL, Kapfhamer D, Sufalko D, Robinson MS, Noebels JL, Burnmeister M: **Mutation in AP-3 in the mocha mouse links endosomal transport to storage deficiency in platelets, melanosomes, and synaptic vesicles.** *Neuron* 1998, **21**:111-122.
32. Feng L, Seymour AB, Jiang S, To A, Peden AA, Novak EK, Zhen L, Rusiniak ME, Eicher EM, Robinson MS *et al.*: **The β 3A subunit gene (Ap3b1) of the AP-3 adaptor complex is altered in the mouse hypopigmentation mutant pearl, a model for Hermansky-Pudlak syndrome and night blindness.** *Hum Mol Genet* 1999, **8**:323-330.
- The mouse coat color mutants, mocha and pearl, are animal models for the human pigmentation and blood platelet disorder, Hermansky-Pudlak syndrome. In a previous study, Kantheti *et al.* [31] had demonstrated that the mocha mouse carries mutations in the gene encoding the δ subunit of AP-3. In this study, the pearl mouse is shown to have mutations in the β 3A subunit of AP-3. Together, these studies establish a role for AP-3 in the biogenesis of lysosome-related organelles such as melanosomes and platelet dense bodies.
33. Dell'Angelica EC, Shotelersuk V, Aguilar RC, Gahl WA, Bonifacino JS: **Altered trafficking of lysosomal proteins in Hermansky-Pudlak syndrome due to mutations in the β 3A subunit of the AP-3 adaptor.** *Mol Cell* 1999, **3**:11-21.
- This study identifies two human patients with Hermansky-Pudlak syndrome that, like the pearl mouse, have mutations in the gene encoding the β 3A subunit of AP-3. Fibroblasts from these patients exhibit increased trafficking of lysosomal membrane proteins via the plasma membrane en route to lysosomes, indicating that a trafficking defect might underlie the defective biogenesis of melanosomes and platelet dense bodies characteristic of this disease.
34. Zizioli D, Meyer C, Guhde G, Saftig P, von Figura K, Schu P: **Early embryonic death of mice deficient in γ -adaptin.** *J Biol Chem* 1999, **274**:5385-5390.
35. Meyer C, Zizioli D, Lausmann S, Eskelinen EL, Hamann J, Saftig P, von Figura K, Schu P: **μ 1A-adaptin-deficient mice: lethality, loss of AP-1 binding and rerouting of mannose 6-phosphate receptors.** *EMBO J* 2000, **19**:2193-2203.
36. Le Borgne R, Alconda A, Bauer U, Hoflack B: **The mammalian AP-3 adaptor-like complex mediates the intracellular transport of**

- lysosomal membrane glycoproteins. *J Biol Chem* 1998, 273:29451-29461.
37. Dell'Angelica EC, Aguilar RC, Wolins N, Hazelwood S, Gahl WA, Bonifacino JS: Molecular characterization of the protein encoded by the Hermansky-Pudlak syndrome type 1 gene. *J Biol Chem* 2000, 275:1300-1306.
 38. Cowles CR, Odorizzi G, Payne GS, Emr SD: The AP-3 adaptor complex is essential for cargo-selective transport to the yeast vacuole. *Cell* 1997, 91:109-118.
 39. Stepp JD, Huang K, Lemmon SK: The yeast adaptor protein complex, AP-3, is essential for the efficient delivery of alkaline phosphatase by the alternate pathway to the vacuole. *J Cell Biol* 1997, 139:1761-1774.
 40. Odorizzi G, Cowles CR, Emr SD: The AP-3 complex: a coat of many colours. *Trends Cell Biol* 1998, 8:282-288.
 41. Huang L, Kuo YM, Gitschier J: The pallid gene encodes a novel, syntaxin 13-interacting protein involved in platelet storage pool deficiency. *Nat Genet* 1999, 23:329-332.
 42. Dettler JC, Zhang Q, Mules EH, Novak EK, Mishra VS, Li W, McMurtrie EB, Tchernev VT, Wallace MR, Seabra MC *et al.*: Rab geranylgeranyl transferase α mutation in the gunmetal mouse reduces Rab prenylation and platelet synthesis. *Proc Natl Acad Sci USA* 2000, 97:4144-4149.
 43. ter Haar E, Harrison SC, Kirchhausen T: Peptide-in-groove interactions link target proteins to the β -propeller of clathrin. *Proc Natl Acad Sci USA* 2000, 97:1096-1100.
 44. Drake MT, Zhu Y, Kornfeld S: The assembly of AP-3 adaptor complex-containing clathrin-coated vesicles on synthetic liposomes. *Mol Biol Cell* 2000, 11:3723-3736.
 45. Shi G, Faundez V, Roos J, Dell'Angelica EC, Kelly RB: Neuroendocrine synaptic vesicles are formed *in vitro* by both clathrin-dependent and clathrin-independent pathways. *J Cell Biol* 1998, 143:947-955.
 46. Rehling P, Darsow T, Katzmam DJ, Emr SD: Formation of AP-3 transport intermediates requires VPS41 function. *Nat Cell Biol* 1999, 1:346-353.
- See annotation [47*].
47. Darsow T, Katzmam DJ, Cowles CR, Emr SD: Vps41p function in the alkaline phosphatase pathway requires homooligomerization and interaction with AP-3 through two distinct domains. *Mol Biol Cell* 2001, 12:37-51.
- These two studies [46*,47*] use both genetics and biochemistry to demonstrate that the yeast VPS41 gene encodes a protein that acts together with AP-3 to form alkaline-phosphatase-containing vesicles, which are transported from the late Golgi to the vacuole.
48. Radisky DC, Snyder WB, Emr SD, Kaplan J: Characterization of VPS41, a gene required for vacuolar trafficking and high-affinity iron transport in yeast. *Proc Natl Acad Sci USA* 1997, 94:5662-5666.
 49. Faundez V, Horng JT, Kelly RB: A function for the AP3 coat in synaptic vesicle formation from endosomes. *Cell* 1998, 93:423-432.
 50. Wang X, Kilian MW: Identification of two new μ -adaptin-related proteins, μ -ARP1 and μ -ARP-2. *FEBS Lett* 1997, 402:57-61.
 51. Dell'Angelica EC, Mullins C, Bonifacino JS: AP-4, a novel protein complex related to clathrin adaptors. *J Biol Chem* 1999, 274:7278-7285.
- See annotation [52*].
52. Hirst J, Bright NA, Rous B, Robinson MS: Characterization of a fourth adaptor-related protein complex. *Mol Biol Cell* 1999, 10:2787-2802.
- These two studies by Dell'Angelica *et al.* [51*], and Hirst *et al.* [52*] describe the identification and preliminary characterization of the mammalian AP-4 complex. This complex is composed of four ubiquitously expressed subunits termed ϵ , β 4, μ 4 and σ 4. AP-4 is shown to be a component of a non-clathrin coat associated with the *trans*-Golgi network.
53. Stephens DJ, Banting G: Specificity of interaction between adaptor-complex medium chains and the tyrosine-based sorting motifs of TGN38 and Igp120. *Biochem J* 1998, 335:567-572.
 54. Aguilar RC, Boehm M, Gorshkova I, Crouch RJ, Tomita K, Saito T, Ohno H, Bonifacino JS: Signal-binding specificity of the μ 4 subunit of the adaptor protein complex, AP-4. *J Biol Chem* 2001, in press.
 55. Hirst J, Lui WWY, Bright NA, Totty N, Seaman MNJ, Robinson MS: A family of proteins with γ -adaptin and VHS domains that facilitate trafficking between the TGN and the vacuole/lysosome. *J Cell Biol* 2000, 149:67-79.
- See annotation [59*].
56. Dell'Angelica EC, Puertollano R, Mullins C, Aguilar RC, Vargas JD, Hartnell LM, Bonifacino JS: GGAs: a family of ADP ribosylation factor-binding proteins related to adaptors and associated with the Golgi complex. *J Cell Biol* 2000, 149:81-93.
- See annotation [59*].
57. Takatsu H, Yoshino K, Nakayama K: Adaptor γ ear homology domain conserved in gamma-adaptin and GGA proteins that interact with γ -synergism. *Biochem Biophys Res Commun* 2000, 271:719-725.
- See annotation [59*].
58. Poussu A, Lohi O, Lehto VP: Vear, a novel Golgi-associated protein with VHS and γ -adaptin 'ear' domains. *J Biol Chem* 2000, 275:7176-7183.
- See annotation [59*].
59. Boman AL, Zhang CJ, Zhu X, Kahn RA: A family of ADP-ribosylation factor effectors that can alter membrane transport through the *trans*-Golgi. *Mol Biol Cell* 2000, 11:1241-1255.
- Each of these papers [55*-59*] independently reports the discovery of the GGAs. All of the studies show that the GGAs localize to the Golgi complex in mammalian cells; in addition, Boman *et al.* [59*] and Dell'Angelica *et al.* [56*] show that they bind to activated ARF, and Hirst *et al.* [55*] and Dell'Angelica *et al.* [56*] show that deleting the GGA genes from yeast causes missorting of the vacuolar hydrolase CPY, implicating the proteins in traffic from the TGN to endosomes.
60. Costaguta C, Stefan CJ, Bensen ES, Emr SD, Payne GS: Yeast Gga proteins function with clathrin in Golgi to endosome transport. *Mol Biol Cell* 2001, in press.
- See annotation [63*].
61. Zhdkanka O, Strand NL, Redmond JM, Boman AL: Yeast GGA proteins interact with GTP-bound Arf and facilitate transport through the Golgi. *Yeast* 2000, 18:1-18.
- See annotation [63*].
62. Black MW, Pelham HR: A selective transport route from Golgi to late endosomes that requires the yeast GGA proteins. *J Cell Biol* 2001, 151:587-600.
- See annotation [63*].
63. Hirst J, Lindsay M, Robinson MS: GGAs: roles of the different domains and comparison with AP-1 and clathrin. *Mol Biol Cell* 2001, in press.
- These four studies [60*-63*] further investigate the role of the GGAs in yeast and show that they are required for the sorting of the prevacuolar syntaxin Pep12p [62*], that yeast as well as mammalian GGAs bind activated ARF [61*], that GGAs are required for α -factor maturation [60*,63*], that GGAs act together with clathrin [60*] and that the VHS and GAT domains are essential for function while the γ -adaptin ear homology domain contributes to GGA function but is not absolutely required [62*].
64. Mao Y, Nickitenko A, Duan X, Lloyd TE, Wu MN, Bellen H, Quirocho FA: Crystal structure of the VHS and FYVE tandem domains of Hrs, a protein involved in membrane trafficking and signal transduction. *Cell* 2000, 100:447-456.
 65. Misra S, Beach BM, Hurley JH: Structure of the VHS domain of human Tom1 (target of myb 1): insights into interactions with proteins and membranes. *Biochemistry* 2000, 39:11282-11290.
 66. Nielsen MS, Madsen P, Christensen EI, Nykjaer A, Gliemann J, Kaspar-Biermann D, Pohlmann R, Petersen CM: The sortilin cytoplasmic tail conveys Golgi-endosome transport and binds the VHS domain of the GGA2 sorting protein. *EMBO J* 2001, 20:2180-2190.
- See annotation [69*].
67. Puertollano R, Aguilar RC, Gorshkova I, Crouch RJ, Bonifacino JS: Sorting of mannose 6-phosphate receptors mediated by the GGAs. *Science* 2001, 292:1712-1716.
- See annotation [69*].
68. Chen HJ, Yuan J, Lobel P: Systematic mutational analysis of the cation-independent mannose 6-phosphate/insulin-like growth factor II receptor cytoplasmic domain. An acidic cluster containing a key aspartate is important for function in lysosomal enzyme sorting. *J Biol Chem* 1997, 272:7003-7012.

69. Zhu Y, Doray B, Possu A, Lehto V-P, Kornfeld S: **Binding of GGA2 to the lysosomal enzyme sorting motif of the mannose 6-phosphate receptor.** *Science* 2001, **292**:1716-1718.
These three studies [66*–68*] demonstrate that the VHS domains of the human GGAs specifically bind acidic-cluster-dileucine sorting signals contained within the cytosolic tails of sortilin [66*], the cation-independent mannose 6-phosphate receptor [67*,68*], and the cation-dependent mannose 6-phosphate [67*]. The acidic-cluster-dileucine signals are required for sorting from the TGN to endosomes [66*]. Expression of a carboxy-terminally truncated GGA causes the mannose 6-phosphate receptors to become trapped in the TGN, indicating that the GGAs mediate their export from the TGN.
70. Phan HL, Finlay JA, Chu DS, Tan P, Kirchhausen T, Payne GS: **The *S. cerevisiae* APS1 gene encodes a homologue of the small subunit of the mammalian clathrin AP-1 complex: evidence for functional interaction with clathrin at the Golgi complex.** *EMBO J* 1994, **13**:1706-1717.
71. Puertollano R, Randazzo PA, Presley JF, Hartnell LM, Bonifacino JS: **The GGAs promote ARF-dependent recruitment of clathrin to the TGN.** *Cell* 2001, **105**:93-102.
This study shows that the GGAs prevent ARF-GAP activity, and demonstrates by mutagenesis that ARF binding and membrane recruitment are coupled. The authors also show that the GGAs are clathrin-associated, that they can bind clathrin *in vitro*, and that they promote its recruitment onto membranes.
72. Stepp JD, Pellicena-Palle A, Hamilton S, Kirchhausen T, Lemmon S: **A late Golgi sorting function for *Saccharomyces cerevisiae* Apm1p, but not for Apm2p, a second yeast clathrin AP medium chain-related protein.** *Mol Biol Cell* 1995, **6**:41-58.
73. Huang KM, D'Hondt K, Riezman H, Lemmon SK: **Clathrin functions in the absence of heterotetrameric adaptors and AP180-related proteins in yeast.** *EMBO J* 1999, **18**:3897-3908.
74. Yeung BG, Phan HL, Payne GS: **Adaptor complex-independent clathrin function in yeast.** *Mol Biol Cell* 1999, **10**:3643-3659.
75. Fernandez-Chacon R, Achiriloaie M, Janz R, Albanesi JP, Sudhof TC: **SCAMP1 function in endocytosis.** *J Biol Chem* 2000, **275**:12752-12756.
76. Andrews J, Smith M, Merakovsky J, Coulson M, Hannan F, Kelly LE: **The stoned locus of *Drosophila melanogaster* produces a dicistronic transcript and encodes two distinct polypeptides.** *Genetics* 1996, **143**:1699-1711.
77. Grigliatti TA, Hall L, Rosenbluth R, Suzuki DT: **Temperature-sensitive mutations in *Drosophila melanogaster*. XIV. A selection of immobile adults.** *Mol Gen Genet* 1973, **120**:107-114.
78. Stimson DT, Estes PS, Rao S, Krishnan KS, Kelly LE, Ramaswami M: ***Drosophila* stoned proteins regulate the rate and fidelity of synaptic vesicle internalization.** *J Neurosci* 2001, **21**:2034-3044.
This study shows that *Drosophila* stoned mutants display reduced endocytosis of synaptic vesicle membranes and depletion and enlargement of synaptic vesicles in presynaptic terminals. The authors also demonstrate that mutations in the stoned locus enhance the endocytic defects in the dynamin mutant, shibire.
79. Fergestad T, Broadie K: **Interaction of stoned and synaptotagmin I in synaptic vesicle endocytosis.** *J Neurosci* 2001, **21**:1218-1227.
This study shows that the *Drosophila* stoned A and stoned B colocalize with components of the endocytic machinery such as AP-2 and dynamin in presynaptic terminals. In addition, stoned mutant flies are found to exhibit impaired endocytosis of synaptic vesicle endocytosis after neurotransmitter release. Finally, transgenic overexpression of synaptotagmin I is shown to restore endocytosis to normal levels, suggesting that the endocytic defects in stoned mutants are due to mislocalization of synaptotagmin I.
80. Fergestad T, Davis WS, Broadie K: **The stoned proteins regulate synaptic vesicle recycling in the presynaptic terminal.** *J Neurosci* 1999, **19**:5847-5860.
81. Stimson DT, Estes PS, Smith M, Kelly LE, Ramaswami M: **A product of the *Drosophila* stoned locus regulates neurotransmitter release.** *J Neurosci* 1998, **18**:9638-9649.
82. Benmerah A, Begue B, Dautry-Varsat A, Cerf-Bensussan N: **The ear of alpha-adaptin interacts with the COOH-terminal domain of the Eps 15 protein.** *J Biol Chem* 1996, **271**:12111-12116.
83. Upadhyaya AB, Lee SH, DeJong J: **Identification of a general transcription factor TFIIAalpha/beta homologue selectively expressed in testis.** *J Biol Chem* 1999, **274**:18040-18048.
84. Martina JA, Bonangelino CJ, Aguilar RC, Bonifacino JS: **Stonin 2: an adaptor-like protein that interacts with components of the endocytic machinery.** *J Cell Biol* 2001, **153**:1111-1120.
This article describes the identification and characterization of two ubiquitously expressed human homologues of *Drosophila* stoned B, named stonin 1 and stonin 2. Stonin 2 binds to eps 15 and intersectin via NPF-motif-EH domain interactions. Like *Drosophila* stoned B, human stonin 2 also binds members of the synaptotagmin family of proteins. Overexpression of stonin 2 inhibits receptor-mediated endocytosis in non-neuronal cells, suggesting that this protein is a component of the endocytic machinery.
85. Salcini AE, Confalonieri S, Doria M, Santolini E, Tassi E, Minenkova O, Cesareni G, Pelicci PG, Di Fiore PP: **Binding specificity and *in vivo* targets of the EH domain, a novel protein-protein interaction module.** *Genes Dev* 1997, **11**:2239-2249.
86. Phillips AM, Smith M, Ramaswami M, Kelly LE: **The products of the *Drosophila* stoned locus interact with synaptic vesicles via synaptotagmin.** *J Neurosci* 2000, **20**:8254-8261.
Drosophila stoned A and stoned B are shown to interact both physically and genetically with synaptotagmin I. Stoned B is found to be associated with a subpopulation of synaptic vesicles, suggesting a possible role for stoned B in a post-endocytic event of the synaptic vesicle cycle. *Drosophila* stoned A and stoned B are shown to interact both physically and genetically with synaptotagmin I. Stoned B is found to be associated with a subpopulation of synaptic vesicles, suggesting a possible role for stoned B in a post-endocytic event of the synaptic vesicle cycle.
87. Zhang JZ, Davletov BA, Sudhof TC, Anderson RGW: **Synaptotagmin I is a high affinity receptor for clathrin AP-2: implications for membrane recycling.** *Cell* 1994, **78**:751-760.
88. Haucke V, Went MR, Chapman ER, Farsad K, De Camilli P: **Dual interaction of synaptotagmin with mu2- and alpha-adaptin facilitates clathrin-coated pit nucleation.** *EMBO J* 2000, **19**:6011-6019.